UNIVERSIDADE DE LISBOA
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Dissertação

Characterization of peripheral blood B cell subpopulations in Rheumatoid Arthritis

Cláudia Cristina Valente Quaresma

Mestrado em Biologia Molecular e Genética

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RESUMO

A artrite reumatoide (AR) é uma doença autoimune crónica, sistémica e de etiologia desconhecida, que afeta cerca de 0,5-1% da população mundial. A AR é caracterizada por uma poliartrite simétrica associada a dor e tumefação de múltiplas articulações, em particular das pequenas articulações das mãos. Embora não exista ainda cura para a AR, sabe-se que um tratamento adequado, assim como um diagnóstico precoce são de extrema importância para o doente e para a sociedade em geral. Se não for corretamente tratada, a AR conduz a destruição óssea e articular. Deste modo, é crucial que a terapêutica seja a mais adequada possível ao estado clínico do doente e implementada na fase inicial do curso da doença.

O tratamento da AR tem como principais objetivos a preservação da função e qualidade de vida do doente, minimizando a dor e sinais inflamatórios, a remissão e controlo das manifestações sistémicas. Os primeiros tratamentos administrados aos doentes com AR são drogas anti-inflamatórias não esteróides e corticóides, úteis no alívio dos sintomas clínicos e na diminuição da inflamação. Contudo, torna-se primordial o uso de drogas antirreumáticas modificadoras da doença como, por exemplo, o metotrexato (MTX), eficaz no controlo da atividade e progressão da doença. Todavia, a introdução das terapias biológicas como as drogas anti-fator de necrose tumoral (anti-TNF) ou o anticorpo monoclonal bloqueador do recetor da interleucina-6 (IL-6R) tocilizumab (TCZ), possibilitou atingir uma melhoria considerável na inibição da progressão da doença.

Várias doenças autoimunes são dependentes de células B, principalmente através da produção de autoanticorpos, tal como a AR. Estudos anteriores revelaram a importância das células B na patogénese da AR através de diversos mecanismos. As células B produzem autoanticorpos, como o fator reumatoide (FR) e anticorpos anti-proteínas citrulinadas (ACPA), que formam complexos imunes que se depositam nas articulações, causando inflamação. Além disso, as células B podem funcionar como células apresentadoras de antigénio e ativar células T, sendo também capazes de produzir citocinas uma vez ativadas e participar na organização de estruturas linfóides secundárias. A descoberta da eficácia da terapêutica de depleção de células B com rituximab (RTX) trouxe não só melhorias na qualidade de vida dos doentes, como também veio reforçar a posição-chave destas células nesta doença autoimune.

O principal objetivo deste trabalho foi a caracterização das subpopulações de células B no sangue periférico de doentes com AR em fase inicial, sem terapêutica e em doentes com AR estabelecida. Adicionalmente, de forma a analisar as possíveis influências do tratamento no desenvolvimento dos processos imunológicos mediados pelas células B na evolução da AR, foi também comparado o efeito dos diversos tratamentos (MTX, anti-TNF e TCZ) no fenótipo das células B e na produção de citocinas e quimiciocinas diretamente relacionadas com a ativação de células B na AR. Para tal, foram incluídos neste estudo, um grupo de doentes com poliartrite não tratada (n=13) com menos de 1 ano de duração da doença, os quais foram classificados como AR iniciais (ERA). Foram também analisados doentes com AR estabelecida sob terapêutica com MTX (MTX, n=17) e MTX pré-biológico (MTX pre-bio, n=29). Dentro do grupo MTX pre-bio, foi realizada uma segunda colheita de sangue em doentes com AR oito meses após terem iniciado tratamento com anti-TNF (n=10) ou TCZ (n=7).

A análise da frequência das subpopulações das células B no sangue periférico foi efetuada usando o sistema de classificação IgD/ CD27 que permite a identificação das principais quatro subpopulações de células B (CD19+): *naïve* (IgD+CD27-), *pre-switch-memory* (IgD+CD27+), *post-switch memory* (IgD-CD27+) e *double-negative* (DN, IgD-CD27-). De forma a poder identificar e caracterizar a subpopulação de plasmócitos no sangue periférico, foi usado o sistema de classificação IgD/ CD38, que identifica os plasmócitos como IgD-CD38++, dentro da região das células B CD19+.

Neste estudo não foram observadas diferenças nas células B totais (CD19+) nos doentes com AR em comparação com os controlos saudáveis. Contudo, quando analisadas as subpopulações de células B, foram observadas alterações nas células B de memória em circulação, nomeadamente um aumento da subpopulação de células B de memória DN (IgD-CD27-) nos doentes com AR estabelecida tratados com MTX e MTX pré-biológico, em relação aos controlos saudáveis.

De modo a caracterizar o fenótipo das células B na fase inicial e estabelecida da AR, foram estudados vários marcadores celulares diretamente relacionados com a ativação e sobrevivência de células B (BAFF-R, TACI e BCMA), ativação celular (HLA-DR, CD69 e CD86), ativação mediada pelo recetor de células B (IgM), diferenciação (CD5), quimiotaxia das células B (CXCR5), apoptose mediada pelo complexo Fas (CD95) e ativação mediada pelo *Toll-like receptor* 9 (TLR9), através da análise da intensidade média da fluorescência (MFI). Adicionalmente, foram quantificados no soro de todos os grupos analisados os níveis

de CXCL13, uma quimiocina importante para as células B, e o CD23 solúvel (sCD23), envolvido na maturação das células B.

O BAFF, uma citocina fundamental na sobrevivência das células B, tem sido sugerida como potencial alvo terapêutico para doenças autoimunes dependentes de células B, como a AR. Esta citocina apresenta 3 recetores: BAFF-R, TACI e BCMA. Neste estudo observou-se um aumento da MFI do TACI em doentes com AR estabelecida sob terapêutica com anti-TNF, em particular na subpopulação de células B *post-switch memory.* Nos restantes recetores do BAFF não foram encontradas diferenças significativas em comparação com os controlos saudáveis. O recetor TACI tem uma função ambígua, pois pode funcionar como recetor de ativação e/ ou de inibição das células B. É possível que o tratamento com anti-TNF induza um aumento da expressão de TACI nas células B de modo a inibir a sua ativação e o desenvolvimento da autoimunidade.

Estudos anteriores têm evidenciado que as células B, uma vez ativadas, aumentam a expressão de marcadores de ativação como o HLA-DR, CD69 e CD86. Neste trabalho, embora não tenham sido observadas diferenças na MFI do CD69 e CD86 nas subpopulações de células B analisadas nos grupos de doentes em comparação com os controlos saudáveis, foi encontrado um aumento significativo na MFI do HLA-DR em doentes com AR estabelecida sob terapêutica com anti-TNF e TCZ. Estudos anteriores demonstraram que as terapêuticas com anti-TNF e TCZ diminuem a infiltração celular observada na membrana sinovial dos doentes com AR. É possível que, devido ao efeito da terapêutica, as células B ativadas que infiltram as articulações regressem ao sangue periférico, justificando deste modo o aumento da MFI do HLA-DR. Foi também observada uma diminuição da MFI do CD86 nos doentes com AR estabelecida tratados com anti-TNF e TCZ em comparação com as colheitas baseline, o que sugere uma inibição da ativação das células B induzida pelo tratamento com imunosupressores.

A apoptose por meio do recetor de morte celular Fas (CD95) desempenha um papel central na manutenção da auto-tolerância imunológica periférica. De facto, alterações nesta via de apoptose foram demonstradas na patogénese de doenças autoimunes como a AR. Neste estudo, valores aumentados de MFI do CD95 foram observados nas células B de memória *post-switch* em doentes com AR estabelecida após o tratamento com anti-TNF e TCZ quando comparados com controlos saudáveis, o que poderá ser uma consequência direta do tratamento como forma de neutralizar a ativação de células B autoreativas.

Estudos efetuados na AR e noutras doenças autoimunes têm revelado alterações nas células B CD5+. Estas células estão associadas à reabsorção óssea através da produção de IL-6, uma citocina que estimula a diferenciação de osteoclastos, as células responsáveis pela erosão óssea. Este marcador também tem sido sugerido como um regulador negativo da ativação de células B. Neste trabalho foi encontrada uma diminuição significativa da frequência de células B CD5+ em todos os grupos de doentes com AR estudados, em comparação com controlos saudáveis. A diminuição da frequência destas células em circulação em todos os grupos de doentes poderá ser resultante do recrutamento de células B CD5+ para a membrana sinovial, onde ocorre o principal processo inflamatório na AR, o que poderá contribuir para a erosão óssea. Além disso, foram observados aumentos dos valores de MFI do CD5 em doentes com AR estabelecida sob terapêutica com anti-TNF e TCZ, que poderão estar relacionados com a função inibitória deste marcador na ativação das células B.

A estimulação de células B através de TLRs também pode ser um mecanismo diretamente relacionado com autoimunidade. O TLR9, em particular, tem sido associado ao desenvolvimento da AR. Sabe-se que a associação do BCR e TLR9 pode ativar as células B autoreativas que reconhecem o DNA CpG endógeno libertado a partir de células apoptóticas e o DNA-CpG IgG dos complexos imunes nas articulações com AR, e induzir a transformação das células B em plasmócitos produtores do FR. Neste estudo, a expressão aumentada de TLR9 foi observada em todas as subpopulações de células B nos doentes com AR estabelecida após o tratamento com TCZ quando comparados com os controlos saudáveis. Além disso, foram observados aumentos nos valores de MFI do TLR9 nas células B *post-switch memory* e células *DN* em doentes tratados com MTX em comparação com os controlos saudáveis. Estas observações sugerem que as células B podem ter mecanismos alternativos para perpetuar a autoimunidade, através da ativação mediada pelo TLR9.

O CXCR5 é um recetor de quimiocina expresso nas células B de recirculação. Este recetor de quimiocina e o seu ligando, CXCL13, estão envolvidos na quimiotaxia de células B, estando regulados positivamente no líquido sinovial dos doentes com AR. Neste estudo, foi observado um aumento dos níveis de expressão (MFI) do CXCR5 nas células B e um aumento dos níveis séricos de CXCL13 em doentes com AR quando comparados com controlos saudáveis, o que pode indicar uma regulação positiva da quimiotaxia de células B na AR e o seu recrutamento para os locais de inflamação, como as articulações.

Durante a ativação de células B, o CD23 membranar (mCD23) é clivado por uma protease associada à célula e libertado como CD23 solúvel (sCD23). Níveis aumentados de sCD23 foram encontrados em doentes com AR, os quais estão diretamente relacionados com a erosão das articulações. Neste estudo, o aumento dos níveis séricos de sCD23 detetados em doentes ERA não tratados em comparação com os controlos saudáveis, poderá indicar uma ativação precoce das células B desde a fase inicial da doença.

Em conclusão, os resultados desta tese suportam a existência de alterações nas células B de memória em circulação na fase estabelecida da AR. Além disso, os níveis de expressão de marcadores celulares podem ser afetados pelos tratamentos com anti-TNF e TCZ, nomeadamente marcadores de ativação (HLA-DR, CD86). Doentes na fase inicial da doença, sem terapêutica apresentam diminuições significativas nas frequências de células B CD5+, expressão elevada de CXCR5 e níveis elevados de CXCL13 e sCD23 no soro em comparação com os controlos saudáveis, o que suporta a hipótese da ativação das células B e o seu envolvimento na patogénese e desenvolvimento da AR desde a fase inicial da doença.

Palavras-chave: Artrite Reumatoide, Células B, Marcadores celulares, Metotrexato, Anti-TNF, Tocilizumab

ABSTRACT

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterized by chronic pain and progressive joint damage. The etiology of RA is unknown and the disease prevalence in the adult population is 0.5-1% worldwide. If left untreated, RA leads to joint destruction, functional disability, comorbidity and reduced life expectancy.

Different effector pathways and cells are involved in the cascade of events leading to the progression and persistence of the disease. B cells play critical roles in RA physiopathology through diverse mechanisms. B cells produce autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), which form immune complexes that deposit in the joints, causing inflammation. Additionally, B cells can function as antigen presenting cells and activate T cells; release cytokines once activated and participate in ectopic lymphoid organogenesis.

The main goal of this work was to characterize B cell subpopulations in the peripheral blood from untreated early rheumatoid arthritis (ERA) patients and established treated RA patients. A full characterization of peripheral blood B cell subpopulations, serum cytokine and chemokine environment was performed.

It was found that established RA patients have disturbances in the frequencies of memory B cell subpopulations in circulation, namely a significant increase in double negative IgD-CD27- B cells. Also, the expression levels of cellular markers can be affected by TNF inhibitors and TCZ treatment, particularly activation markers (HLA-DR, CD86). Furthermore, untreated ERA patients have significantly decreased frequencies of CD5+ B cells, elevated CXCR5 expression and higher serum CXCL13 and sCD23 levels in comparison with controls, which support an early B cell activation in RA pathogenesis.

In conclusion, alterations in the mechanisms associated with B cells' physiopathology are observed since early RA that might be modulated by treatment with TNF inhibitors and TCZ at later stages of the disease.

Keywords: Rheumatoid Arthritis, B cells, Cellular markers, Methotrexate, TNF-inhibitors, Tocilizumab

LIST OF ABBREVIATIONS

ACPAs	Anti-citrullinated protein antibodies
AID	Activation-induced cytidine deaminase
Anti-CCP	Anti-cyclic citrullinated peptide
APCs	Antigen-presenting cells
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
CDR	Complementary determining region
CSR	Class switch recombination
BCR	B cell receptor
CRP	C-reactive protein
CXCL13	Chemokine (C-X-C motif) ligand 13
CXCR5	Chemokine (C-X-C motif) receptor 5
DAS 28	Disease activity score of 28 joints
DCs	Dendritic cells
DMARDs	Disease modifying anti-rheumatic drugs
EA	Early arthritis
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
ERA	Early rheumatoid arthritis
EULAR	European League Against Rheumatism
FDC	Follicular dendritic cell
GC	Germinal center
HLA	Human leukocyte antigen
ICOS	Inducible Costimulator Molecule
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
MFI	Mean of intensity fluorescence
MHC	Major histocompatibility complex
MTX	Methotrexate
NC	Normal controls
NSAIDs	Nonsteroidal anti-inflammatory drugs

OA	Osteoarthritis
PAD	Peptidylarginine Deiminase
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RTX	Rituximab
SE	Shared epitope
SF	Synovial fluid
SLE	Systemic lupus erythematous
SMZ	Splenic marginal zone
TACI	Transmembrane activator and calcium modulator cyclophilin ligand interactor
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	TNF receptor
Tregs	Regulatory T cells

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I. INTRODUCTION

1. Rheumatoid Arthritis

1.1. Definition

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterized by chronic pain and progressive joint damage. The disease prevalence in the adult population is 0,5-1% worldwide. RA onset usually occurs between 30-50 years of age and it is more frequent in women, suggesting a specific hormonal component association. RA is associated with a high risk of functional disability, a diminished quality of life and increased mortality when compared to healthy individuals [1, 2]. Risk factors such as smoking, infectious agents (e.g., Epstein-Barr virus, cytomegalovirus) and their products, environment, obesity can contribute to a worse prognosis [2, 3].

In general, RA is clinically recognized as an inflammatory process affecting joints, but the presence of extra-articular manifestations makes it a systemic disease with different clinical patterns. RA affects mainly the small joints such as those of the hands, wrists and feet and, if not properly treated, leads to bone and cartilage destruction with complete loss of joint integrity [1, 2]. RA is responsible for high levels of functional impairments: 20 to 30% of people with RA become permanently work-disabled within three years of diagnosis and 50% after ten years [4-6].

For years, RA diagnosis was established according to the 1987 American College of Rheumatology (ACR) criteria [7] and at least four of the criteria had to be present for a positive classification as RA. Morning stiffness lasting at least 45 minutes, fatigue and symmetrical inflammation of large and small joints are the most common initial symptoms of RA. Recently, RA diagnosis is defined according to the 2010 ACR/ European League Against Rheumatism (EULAR) classification criteria (appendix 1) [8, 9].

1.2. Etiology and pathophysiology

The etiology of RA is unknown. Different effector pathways and cells are involved in the cascade of events leading to the progression and persistence of the disease [1, 10, 11].

The inflammatory process in RA synovium (Figure 1) is initiated when an unknown antigenic trigger induces the development of an inflammatory autoreactive response. Antigen-presenting cells (APCs) exhibit antigenic processed peptides to T cells, interacting through

the major histocompatibility complex (MHC) – T-cell receptor (TCR) and costimulatory signals via CD28-B7 family receptor CD80/86 [12, 13].

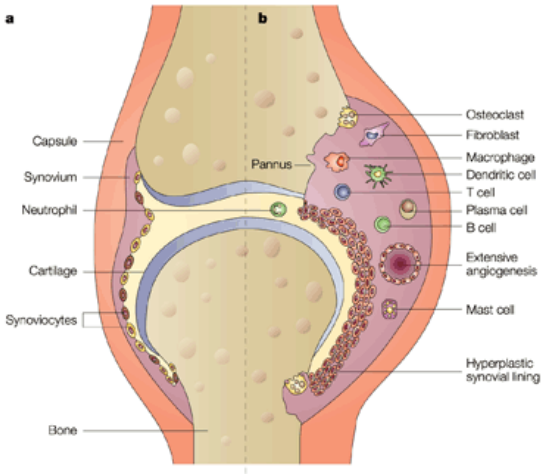

Figure 3. Representative scheme illustrating the differences between healthy (a) and rheumatoid joints (b). Adapted from[14]

B cells can function both as antibody producing cells and as efficient APCs[15]. In RA, autoreactive B cells produce autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptides (anti-CCP)[16], which are able to form immune complexes that deposit in the joints[16, 17]. These immune complexes activate monocytes through the low-affinity IgG receptor FcγRIIIa, leading to the production and release in synovial tissue of several proinflammatory cytokines such as tumor necrosis factor (TNF)[17], interleukin (IL)-1β and IL-6[18]. Activated synovial fibroblasts promote the expression of cell-adhesion molecules such as VCAM1 that contribute to an increase of cellular infiltration in the joints[19]. Additionally, the local production of metalloproteinases (MMP) such as MMP3 or MMP9, as well as the production of receptor activator for nuclear factor κB ligand (RANKL) increases cartilage and bone destruction [20, 21]. The inflamed synovial membrane begins to grow irregularly, forming invasive “pannus” tissue, which consequently invades and destroys cartilage and bone. RA pannus is mainly composed by mononuclear cells and fibroblasts. The inflammatory process is mediated by the activation of intracellular signaling pathways that stimulate the production and the release of multiple cytokines, chemokines, grow factors, proteinases and adhesion molecules that further increase joint erosion. In late-stages of RA, the pannus becomes fibrotic, minimally vascularized, with collagen fibres overlying articular cartilage [21-24].

1.3. Genetic predisposition and environmental risk factors

Rheumatoid Arthritis involves a complex interplay between environmental and genetic risks factors [25]. Heritability studies suggest that 60% of predispositions to RA are explained by genetic factors, leaving the remaining 40% to environmental and stochastic factors [25-27].

The genetic association in RA is due to the histocompatibility leucocyte antigen (HLA) DR genes, which reside in the MHC and participate in antigen presentation [28-30]. The risk for developing RA is related to the presence of specific alleles of the class II gene HLA-DRB1 [31] that encode a conserved sequence of aminoacids, the shared epitope (SE), in the third hypervariable region (HVR3) of the class II DRβ1 chain [30, 31]. This sequence is found in multiple RA-associated DR genes, including DR1, DR4, and DR14. The location of SE on MHC molecules suggests that it might have a role on the ability of HLA-DR to bind and present specific arthritogenic peptides, which might cause RA. In inflamed joints, MHC class II-dependent T cell activation by APCs (B cells, monocytes, dendritic cells) is considered a major driver of the disease, which reinforces the relevance of adaptive immunity in RA pathogenesis [28-31].

The environmental risk factors in RA include female gender, age, a previous family history of RA, smoking, obesity and some infectious agents [3, 32-35].

The best established environmental factor for RA is smoking. A study that included 13 monozygotic twin pairs discordant for RA and smoking, revealed that the smoker was the one who developed the disease in 12 out of 13 pairs [36]. Furthermore, additional studies have also indicated an association between smoking and autoantibody production, namely with anti-citrullinated protein antibodies (ACPA). The observation that RA associated autoantibodies RF and ACPA may be present for more than 10 years before disease onset suggests that risk factors are active several years prior to the development of the disease [26, 33, 36, 37].

Infectious agents such as Epstein-Barr virus [3], cytomegalovirus [38], Proteus species [39], *Mycobacterium tuberculosis* [40], parvovirus B19 [41], *Escherichia coli* and their products (e.g. heat-shock proteins (HSP)) [42] can also be considered risk factors. These associations have been supported by increased antibody titers against the infectious organism present in RA patients and the possibility of molecular mimicry [3, 38-42].

1.4. Prognosis and treatment

In early stages of RA, predictors such as high functional impairment, early involvement of several joints, high erythrocyte sedimentation rate or C-reactive protein levels at disease

onset, positivity for autoantibodies (RF, ACPA) [37, 43] and early radiographic changes can be related to a worse disease prognosis. The presence of specific alleles that encode the susceptibility epitope on MHC molecules might also influence the severity of the disease, since the risk of extra-articular and erosive disease is greater if the patients are homozygous [28, 44]. An adequate RA treatment is most important for the patient and the society. Uncontrolled active RA causes disability, decreased quality of life, and increases comorbidity, which results in loss of employment, high medical and social costs, and substantial morbidity and mortality. This impact of RA ultimately justifies expensive treatment [5, 25]. Although no cure has been found for RA, it is clear that establishing a diagnosis as early as possible and immediate treatment are the basis for a successful management of these patients [25].

The main goals of RA treatment are the preservation of function and quality of life, minimizing pain and inflammation, remission of symptoms and control of systemic manifestations [24, 45-48]

Treatment options are divided in three main classes: 1) nonsteroidal anti-inflammatory drugs (NSAIDs); 2) corticosteroids; and 3) disease modifying anti-rheumatic drugs (DMARDS) (synthetic and biological) [49, 50].

NSAIDs are efficient in relieving RA symptoms, but ineffective in the disease course. Corticosteroids are suppressors of the inflammatory response and previous studies have confirmed that are able to decrease the progression of the RA [51-54].

DMARDs have demonstrated efficiency in managing disease activity by the suppression of the inflammation [55, 56]. Synthetic DMARDs such as methotrexate (MTX) [57], leflunomide, sulfasalazine and hidroxychloroquine are widely used in RA and have proven to be highly beneficial in decreasing inflammation and joint damage [58]. Combinations of DMARDs have also proven efficiency [59-61]. Nevertheless, despite being treated some patients still have persistent disease activity and often present side effects such as gastrointestinal and pulmonary toxicity, headaches, fatigue and less commonly bone marrow suppression [58]. In these cases, when patients do not respond favorably to synthetic DMARDs, treatment with biologic DMARDs must be initiated (Figure 2).

TNF-inhibitory agents are the first line biologic treatment. Infliximab [62, 63], certolizumab [64], adalimumab and golimumab are monoclonal antibodies that target and neutralize circulating and synovial TNF [60]. Etanercept is a fusion protein that functions as a decoy receptor that binds to TNF and lymphotoxin (LT) family members)[50, 65]. Studies have shown that monotherapy with these drugs is effective [60]. Other biological agents include

abatacept, a fusion protein composed of the Fc region of IgG1 fused to the extracellular domain of cytotoxic T-lymphocyte antigen (CTLA)-4 that binds to the CD80/ CD86 molecule and [66, 67] inhibits T cell activation; tocilizumab, an IL-6 receptor antibody antagonist [18, 68]; and anakinra, an IL-1 receptor antagonist that has only a moderate therapeutic effect [69].

Additionally, RA treatment can also be achieved with rituximab (RTX), a monoclonal antibody that selectively depletes B cells. RTX is chimeric monoclonal IgG1 anti-CD20 antibody whose efficiency in RA treatment has brought a new interest to the role of B cells in RA pathogenesis [70-73].

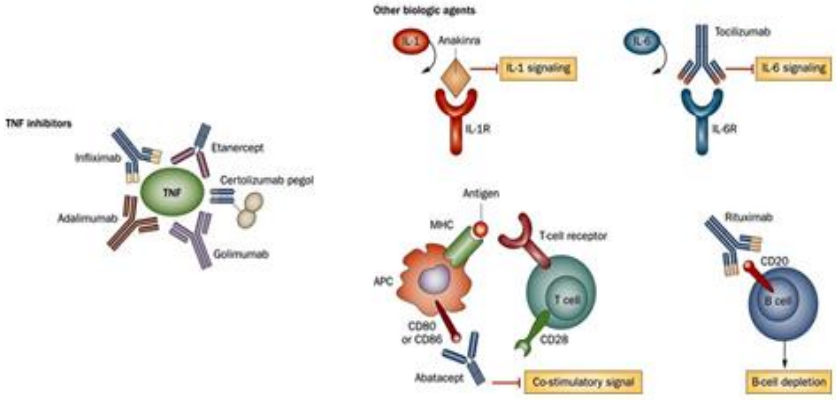

Figure 2. Summary of biologic treatments used in rheumatoid arthritis. Adapted from [74]

2. B cells

2.1 Origin, development and function

B lymphocytes are white blood cells (6-10µm) whit a dense nucleus and little cytoplasm that express a membrane-bound Ig and originate and mature in the bone marrow. In humans, B cells correspond to 5-15% of total lymphocytes ($2X10^2$). These cells are important mediators of humoral immune response, by producing and secreting proteins called immunoglobulins (Ig) or antibodies. B cells can also function as APCs and secrete cytokines that can further activate T cells and contribute to the development of an effective immune response [75, 76].

In humans, during fetal development, B cells are generated in the fetal liver and after birth, bone marrow assumes this function and continues throughout life. B cells arise from a lymphoid stem cell in the bone marrow and precede through several maturation stages, during which they express different cell surface markers. The B cell progenitor (pro-B cell) is the earliest distinctive B-lineage maturation stage. Pro-B cells proliferate in the bone marrow and differentiate into precursor B cells (pre-B cells). Pre-B cell stage is followed by an immature B cell stage that is not fully functional. B cells leave the bone marrow as naïve B cells when maturation is achieved by co-expression of IgM and IgD on their surface [75, 77-81]. The B cell differentiation stages in the bone marrow correspond to the antigen-independent phase of B cell development [81, 82]. Once they leave the bone marrow, naïve B cells, which have never encountered an antigen, circulate in the blood and lymphatic systems and are carried to secondary lymphoid organs (lymph nodes, spleen, Peyer's Patches), where they further differentiate. After encountering an antigen, naïve B cells transform into large B-blasts and may follow two different pathways. Some cells proliferate and differentiate into short-living IgM producing plasma cells and a minority of B-blasts will differentiate to form germinal centers (GC) [82]. In GC reactions, B cells differentiate either into memory B cells or plasma cells (Figure 3).

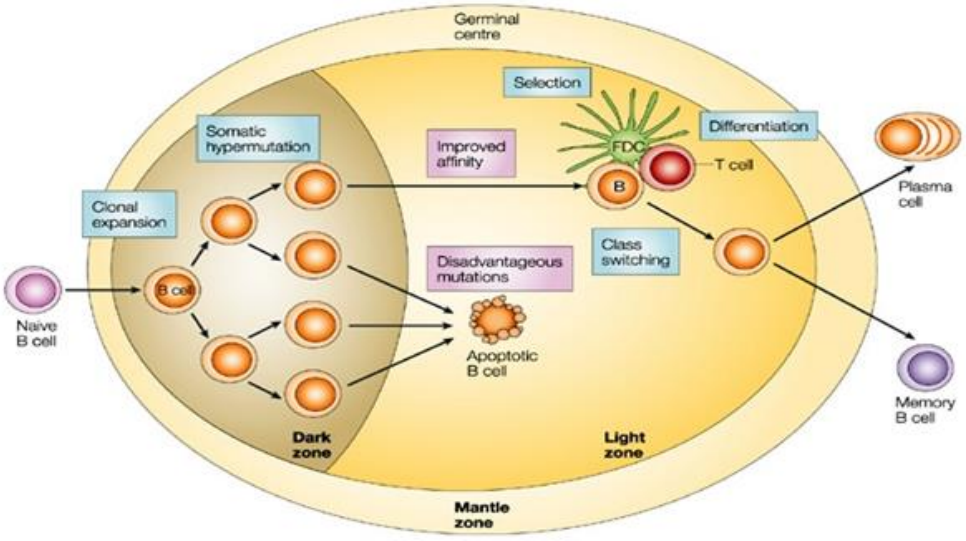

Figure 3: B cells development adapted from [83]

Since an antigen is required for B cell activation and differentiation in the periphery, this stage comprises the antigen-dependent phase of B cell development. In the absence of antigen-induced activation, naïve B cells in the periphery become apoptotic [84].

2.2. Antibodies

Antibodies, also called immunoglobulins, are the antigen-binding proteins secreted by plasma cells, present on the B cell membrane or in circulation. Antibodies can eliminate pathogens by several mechanisms such as opsonization, complement activation and direct lysis of bacteria and/or neutralization of virus and toxins to prevent their entry into host cells [77, 84]. An antibody molecule has a common structure of four peptide chains, two identical light (L) chains and two identical heavy (H) chains and has five major classes based on the diversity of their heavy chain, namely IgM, (μ), IgG (γ), IgA (α), IgD (δ) and IgE (ε), each one with different structural and functional properties [77, 85, 86]. Antibody diversity is achieved by V(D)J recombination (V-variable, D-diversity and J-joining), somatic hypermutation (SHM) and class or isotype-switching [84, 86].

The V(D)J recombination is an ordered site-specific DNA rearrangement process that occurs in developing lymphocytes in the BM, in which V(D)J gene segments are randomly combined at recombination signal sequences (RSS) [84, 87]. RSS are recognized by an endonuclease coded by recombination activating gene (*RAG*)-*1* and *RAG-2*, responsible for DNA double-stranded breaks following the 12-23 bp rule [84, 86]. The diversity generated by this process is further increased by terminal deoxynucleotidyl transferase (TdT) that adds nucleotides to gene segments junctions [84, 86]. SHM is a mechanism that occurs within GC in secondary lymphoid organs and involves the introduction of point mutations, mainly nucleotide substitutions, as well as occasional deletions and duplications at a very high rate into the DNA of heavy and light chain variable region genes, at CDR [86]. This differential selection is due to an increase in antibody affinity for an antigen, a process known as affinity maturation. CSR is an isotype switching deletional DNA recombination process that occurs in mature B cells and consists of replacing an expressed heavy-chain constant-region gene, usually IgM, with another one of a different biological function – IgG, IgA or IgE [86]. During this process, only 10 the effector functions of the antibody are changed. SHM and CSR are mediated by activation-induced cytidine deaminase (AID) that promotes the recombination event [88-90].

3. B cells and rheumatoid arthritis

For decades, RA was considered a T-cell driven disease. In the rheumatoid synovium, accumulation of CD4+ T lymphocytes suggested that macrophages could be activated by T cells, specifically T helper (Th)-1, to produce proinflammatory cytokines such as TNF, IL-1β and IFNγ [91-93].

In patients with RA, the increase of IL-17 levels in synovial fluid [94] suggests the involvement of Th17 cells and this association is directly proportional to the severity of joint

destruction [23, 95]. In fact, the percentage of Th17 cells is increased in RA synovial fluid [96]. These facts are supported by studies performed in animal models of arthritis that demonstrate that a local expression of Il-17 in mouse joints results in acute inflammation, while the incidence and severity of arthritis is markedly attenuated in IL-17-deficient mice [97, 98]. Contrarily to Th1, Th2, and Th17 cells, regulatory T cells (Tregs) CD4+CD25+ are characterized by low proliferative capacity upon triggering the T cell receptor (TCR) and by their ability to suppress CD4+ and CD8+ T-cell immune responses [99]. Several studies have proposed that Tregs are severely impaired in autoimmune rheumatic diseases, suggesting that in fact a breakdown of Treg-mediaded peripheral tolerance may have occurred [99-101]. Despite all the observations that support a role for T cells in RA development, the disappointing results obtained with anti-CD4 therapy in humans reinforced the notion that perhaps other parts of the inflammatory process of RA should be better studied and understood [50, 102, 103].

Although the cause that triggers RA autoimmune process remains unknown, it has been demonstrated that several types of cells from both innate and adaptative immune system actively participate and form complex networks of cell-cell interactions that contribute to the development and chronicity of synovitis and inflamation on RA [104, 105]. Several studies have pointed to B cell function as a critical factor in the development of RA [85, 106-108]. In RA, B cells are responsible for the production of autoantibodies [109], which include antibodies against cartilage components (anti-type II collagen or anti-CII; anti-human cartilage glycoprotein 39 or anti-gp39); enzymes (anti-glucose-6-fosfato-isomerase or anti-GPI, anti-enolase); nuclear proteins (anti-RA33) and stress proteins. RF and ACPAs are, however, the autoantibodies most specifically associated with this disease. RF are autoantibodies that directly bind to the Fc portion of normal human IgG and ACPAs are autoantibodies that recognize peptides or proteins containing citrulline, a non-standard aminoacid generated by the post-translational modification of arginine by peptidylarginine deiminase (PAD) enzymes, in a process known as citrullination. RF and ACPAs can form immune complexes that deposit in the joints, activate complement and cause inflammation [110]. The clinical usefulness of RF and ACPAs has been acknowledged due to their good diagnostic sensitivity and prognostic value, but the clinical significance and pathogenic roles of the other autoantibodies are unknown. Furthermore, the association between high titer of RF and worse prognosis indicate that RF may have an important role in the pathogenesis of RA, although there are RA patients seronegative for RF who also manifest the disease. Moreover, ACPAs-positive RA patients have a higher cellular infiltration in RA synovium and these autoantibodies are also helpful to predict the outcome of patients with undifferentiated arthritis [37, 109, 111].

B cells are also very efficient APCs that activate T cells through the expression of costimulatory molecules [112, 113]. A B cell is considered autoreactive when its B cell receptor (BCR) targets a self-antigen. In RA, B cells have the ability to present processed self-antigens to T cells, thus allowing the development of an autoreactive immune response. Indeed, RF+ B cells play an important role in antigen presentation and in the induction of T cell activation [12, 114, 115].

B cells can also function as cytokine and chemokine-producing cells that promote leukocyte infiltration in the joints and the formation of ectopic lymphoid structures [116, 117], thus aggravating angiogenesis and synovial hyperplasia [15, 118].

The efficacy of B cell depletion therapy with RTX reinforced the relevance of B cells in RA pathogenesis [119].

II. AIMS

The main goals of the present work were:

- To characterize B cell subpopulations in the peripheral blood from untreated early rheumatoid arthritis (ERA) patients and established treated RA patients.
- To analyze the cytokine and chemokine environment directly related with B cell activation since early RA.
- To compare the effect of treatment options (methotrexate-MTX, TNF-inhibitors and tocilizumab-TCZ) on B cell phenotype and cytokine production in ERA and established RA patients.

III. MATERIALS AND METHODS

Patients

Blood samples were collected from 13 consecutive patients with untreated polyarthritis (Rheumatology Department, Hospital de Santa Maria, Lisbon) of < 1 year disease duration. After a minimum follow-up of 3 months, the patients fulfilled the 2010 ACR/ EULAR criteria for RA and were classified as early RA (ERA). In addition, blood samples from 17 patients with established RA treated with methotrexate (MTX); 29 patients with established RA treated with MTX before starting biologic DMARD therapy (MTX pre-biologic, MTX pre-bio); 10 patients under treatment with TNF inhibitors and 7 patients under tocilizumab (TCZ) treatment were also collected for comparison (Rheumatology Department, Hospital de Santa Maria, Lisbon). Furthermore, blood samples from 15 healthy donors were also collected and processed for comparison.

This study was approved by the local ethics committee (Comissão de Ética do Hospital de Santa Maria), and all patients and healthy donors signed an informed consent form. Patient care was conducted in accordance with standard clinic practice, and the study was performed in accordance with the Declaration of Helsinki (2008).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from 40 ml heparinized whole blood following density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Sweden).Cells were washed twice in 1X phosphate buffered saline (PBS) and cellular viability was estimated with 0.4% Trypan blue (Sigma, USA).

Flow Cytometry

Immunophenotyping of B cells was performed in PBMC samples ($1X10^6$ cells/ sample) using matched combinations of anti-human murine monoclonal antibodies (mAbs) conjugated to FITC, phycoerytrin (PE), peridinin chlorophyll protein (PerCP)-Cy5.5, allophycocyanin (APC), PE-Cy7, eFlour 450 and APC-eFluor780.Combinations of anti-CD19 conjugated to PerCP-Cy5.5 or APC, anti-IgD conjugated to PE-Cy7 or FITC, anti-CD27 conjugated to eFluor450 or FITC, anti-CD38 conjugated to APC-eFluor780, anti-BAFF-R conjugated to PE, anti-TACI conjugated to APC, anti-CD86 conjugated to PE, anti-CD69 conjugated to PerCP or APC, anti-IgM conjugated to PE, anti-CD5 conjugated to APC, anti-CXCR5 conjugated to

PE, anti-HLA-DR conjugated to APC, anti-CD95 conjugated to APC, anti-BCMA conjugated to PE and anti-TLR9 conjugated to APC were used. All antibodies were purchased from BD Pharmingen (USA), eBioscience (USA) and R&D Systems (United Kingdom). For cell surface stainings, PBMC were incubated with antibodies during 30 minutes, in the dark, at 4ºC. For TLR9 intracellular staining, PBMC were fixed during 20 minutes at room temperature with IC Fixation Buffer (eBioscience, USA), permeabilized with 1X Permeabilization Buffer (eBioscience, USA) and stained according to eBioscience intracellular antigen staining protocol. A total of 50.000 cells/ sample gated in CD19+ B cells were acquired with LSR Fortessa (BD). Data were analyzed with FlowJo (TreeStar, Stanford University, California, USA). All samples were acquired on the same day of the staining protocol.

ELISA

The B-lymphocyte chemoattractant (BLC) also known as C-X-C motif chemokine 13 (CXCL13) and the soluble form of CD23 (sCD23) were quantified in serum samples from all groups by enzyme-linked immunosorbent assay (ELISA) (R&D systems, United Kingdom), according to the manufacturer's instructions. Samples were analyzed using plate reader Infinite M200 (Tecan, Switzerland).

Statistical analysis

Statistical differences were determined with GraphPad Prism (GraphPad, San Diego, USA). For populations that did not follow a Gaussian distribution, non-parametric tests were used. The Mann-Whitney test was used for comparisons between 2 independent groups. For comparisons between 3 or more groups, the Kruskal-Wallis and Dunn's multiple comparison tests were used. The Wilcoxon matched pairs test was used for comparisons between 2 paired groups. Correlation analyses were performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

IV. RESULTS

1. Clinical characterization of patients

A group of untreated polyarthritis patients (n=13) with less than 1 year of disease duration and classified as early RA (ERA) according to the 2010 ACR/ EULAR criteria after a minimum follow-up of 3 months was included in this study. ERA patients had a mean ± standard deviation age of 58±14 years old, 85% were female, 69% were RF positive, 62% were anti-CCP positive and DAS28 score was 4.1±2.0. A group of established RA patients under MTX treatment (n=17) with a mean age of 55±14 years old, 81% female and a DAS28 of 2.5±1.2 was also included. Furthermore, a group of established RA patients under MTX treatment pre-biological therapy (MTX pre-bio, n=29) with a mean age of 57±11 years old, 83% female and a DAS28 of 5.1±1.3 was also analyzed. From the MTX pre-bio group, a second blood collection was performed to RA patients that had either initiated treatment with TNF-inhibitors (n=10) or with tocilizumab (TCZ) (n=7), after an average follow-up of 8 months of treatment. In addition, blood samples were collected from age and sex-matched healthy donors (n=15). The clinical information and data from all patients and healthy controls included in this study is indicated in Table 1.

Table 1. Clinical information of untreated ERA and established RA patients treated with MTX, MTX pre-bio, TNF inhibitors or TCZ.

	Controls (n=15)	ERA (n=13)	MTX (n=17)	MTX pre-bio (n=29)	TNF (n=10)	TCZ (n=7)
Age (years)	52±7	58±14	55±14	57±11	56±15	61±10
Sex (% female)	73	85	81	83	80	83
Disease duration (years)	NA	≤ 1	7±6	13±12	14±13	8±4
CRP (mg/dl)	ND	1.4±2.1	0.5±0.6	1.1±1.2	1.9±2.5	0.1±0.1**#&
ESR (mm/1st hour)	ND	44±26	23±22	32±22	47±39	6±5**#&
VAS	NA	40±33	38±34#	69±21	62±21	63±13
DAS28	NA	4.1±2.0	2.5±1.2#	5.1±1.3	4.2±1.5	2.3±0.7#
Swollen joints	NA	4±4	1±2#	7±6	2±3	1±1
Tender joints	NA	5±5	2±3#	10±8	6±5	1±1#
RF (+) %	ND	69	77	74	78	50
Anti-CCP (+) %	ND	62	63	68	38	67

ERA - Early Rheumatoid Arthritis; RA - Rheumatoid Arthritis; CRP - C-reactive protein; ESR - Erythrocyte Sedimentation Rate; VAS – Visual Analogue Scale; DAS28 – Disease Activity Score of 28 joints; RF – Rheumatoid Factor; Anti-CCP – anti-cyclic citrullinated peptide; NA – not applicable; ND – not determined. Values are represented as mean ± standard deviation.

** $p < 0.05$ in comparison with ERA # $p < 0.05$ in comparison with MTX pre-bio & $p < 0.05$ in comparison with TNF

2. Classification of B cell subpopulations

To analyze the frequency of B cell subpopulations in the periphery, B cells were classified using the IgD/ CD27 classification system that allows the identification of four main B cell subsets (gated in CD19): naïve B cells (IgD+CD27-), pre-switch-memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double-negative (DN, IgD-CD27-) B cells (appendix 2). A second classification system based on IgD/ CD38 (gated in CD19) was also used to identify circulating plasmablasts, defined as IgD-CD38++. The IgD/ CD38 classification system allows the identification of six B cell subpopulations: transitional (IgD+CD38++), naïve B cells (IgD+38+), IgD+ memory (IgD+CD38-), resting memory (IgD-CD38-), post-GC memory (IgD-CD38+) B cells and plasmablasts (IgD-CD38++) (appendix 2). Although an analysis with both classification systems has been performed in the present work to define B cell subpopulations, the results presented in this thesis are based on IgD/ CD27 classification system (except plasmablasts) in order to compare with previous results obtained by our group in which IgD/ CD27 classification was used [120].

2.1. Established RA patients have alterations in the frequency of memory B cell subpopulations in peripheral blood

The analysis of the frequency of total CD19+ B cells has revealed that no significant differences were found in ERA and established RA patients treated with MTX, MTX pre-bio, TNF inhibitors or TCZ when compared to healthy controls. However, when analyzing B cell subpopulations, it was observed that established RA patients treated with MTX and MTX pre-bio had significantly increased frequencies of DN (IgD-CD27-) B cells in comparison to controls ($p < 0.05$). Furthermore, no significant differences were observed in the remaining B cell subpopulations analyzed (naïve, pre-switch memory and post-switch memory B cells) in all groups (Figure 4). Importantly, no significant correlations were found between the frequencies of all B cell subpopulations with age or with disease activity (DAS28) in all groups studied (data not shown).

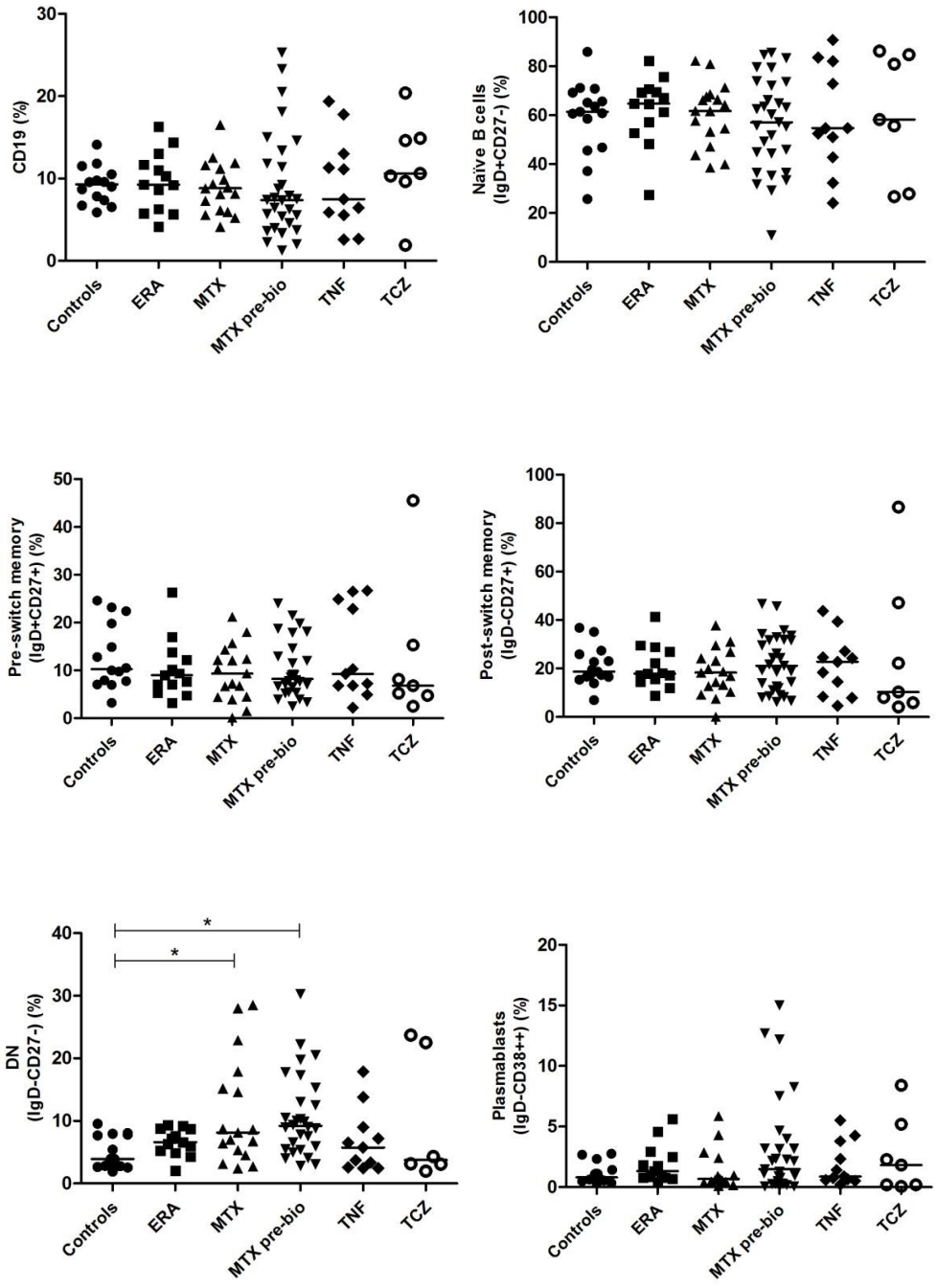

Figure 5. Frequency of B cell subpopulations in ERA and established RA patients treated with MTX, MTX pre-bio, TNF inhibitors and TCZ in comparison with healthy controls. Lines represent median percentage values.

* $p < 0.05$ in comparison with Controls.

3. B cell markers and effect of treatment in comparison with healthy controls

In this study the expression of several cellular markers was analyzed by mean fluorescence intensity (MFI) to characterize B cell phenotype in circulation in untreated early and

established treated RA patients, according to their function. Thus, BAFF-R, TACI and BCMA were studied to analyze the expression of BAFF receptors on B cells; CD69, CD86 and HLA-DR were studied to analyze B cell activation; CXCR5 was studied to analyze B cell chemotaxis; CD95, also known as Fas receptor (FasR), was studied to analyze Fas-mediated apoptosis; IgM was analyzed as a component of the BCR; CD5 was studied to analyze B cell differentiation and TLR9 was studied to analyze the main TLR expressed by B cells. The MFI values obtained for all cellular markers were considered in all B cell subpopulations, except in plasmablasts (IgD-CD38++), due to the low number of events obtained during data acquisition and analysis.

3.1. TACI expression increases on memory B cell subpopulations after treatment with TNF inhibitors, but no changes occur in BAFF-R and BCMA MFI values in all B cell subsets

B cell activating factor (BAFF) is an important cytokine for B cell activation and survival [108, 121, 122] that binds to three receptors: BAFF-R, TACI and BCMA. While no significant differences were found in BAFF-R (Table 2) and BCMA (Table 3) MFI levels in all groups analyzed in all B cell subpopulations, TACI MFI was significantly increased in total CD19+ B cells in established RA patients after treatment with TNF inhibitors in comparison with controls, namely in post-switch memory *(p* = 0.0170) and double negative B cells *(p* = 0.0084) (Table 4). Furthermore, TACI MFI levels were also significantly increased in double negative B cells of ERA patients when compared to controls *(p* = 0.0138).. Moreover, no significant differences were observed in the frequencies of BAFF-R+ B cells in all B cell subpopulations in all groups analyzed (data not shown).

Table 2. MFI values of BAFF-R on B cell subpopulations. Represented values are mean ± standard deviation (SD).

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	2012±1616	1602±1131	1793±1557	2457±1799	2702±1373	1813±1235
Naïve (IgD+27-)	1897±1763	1580±1148	1826±1589	2482±1868	2643±1373	1822±1314
Pre-switch memory (IgD+27+)	2118±1779	1751±1190	2087±1763	2392±1839	2839±1430	1930±1232
Post-switch memory (IgD-27+)	1901±1457	1602±1122	1736±1550	2388±1751	2823±1486	1523±1140
DN (IgD-27-)	2045±1491	1589±1143	1656±1446	2475±1909	2571±1449	1498±1230

Table 3. MFI values of BCMA on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	13±131	26±78	44±112	105±108	58±124	30±95
Naïve (IgD+27-)	29±33	5±27	34±126	30±92	29±153	7±36
Pre-switch memory (IgD+27+)	24±207	98±122	83±202	139±158	70±137	40±154
Post-switch memory (IgD-27+)	109±109	131±57	122±75	169±75	131±61	136±139
DN (IgD-27-)	81±88	111±57	111±74	127±64	103±65	102±112

Table 4. MFI values of TACI on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	181±309	321±394	222±276	202±297	400±249*****	473±490
Naïve (IgD+27-)	219±425	231±352	153±298	178±350	344±250	452±562
Pre-switch memory (IgD+27+)	381±288	546±452	462±335	374±449	733±629	928±1060
Post-switch memory (IgD-27+)	178±217	459±415	264±374	275±380	486±287*****	410±358
DN (IgD-27-)	137±208	464±373*****	333±289	285±357	415±209*****	305±88

* $p < 0.05$ in comparison with Controls

3.2. TNF inhibitors and TCZ treatment increase the expression of the activation marker HLA-DR on B cells, but have no effect on CD69 and CD86

In all groups studied, no significant differences were found in CD69 and CD86 MFI levels in all B cell subpopulations (Tables 5 and 6, respectively). Moreover, although no significant differences were observed in the frequencies of HLA-DR+ B cells in all groups (data not shown), alterations were found in HLA-DR MFI values in established RA patients treated with TNF inhibitors and TCZ in comparison with healthy controls and/ or in comparison with established RA treated with MTX pre-bio (Table 7). Indeed, it was observed that HLA-DR MFI values were increased in total CD19+ B cells, naïve, post-switch memory and DN B cells in established RA patients after treatment with TNF inhibitors in comparison with controls ($p < 0.05$). After TCZ treatment, HLA-DR MFI values were increased in total CD19+ B cells, naïve, pre-switch memory and DN B cells ($p < 0.05$). Additionally, both TNF inhibitors and TCZ treatments induced a significant increase in HLA-DR MFI values in total CD19+ B cells when compared with MTX pre-bio group ($p < 0.05$).

Table 5. MFI values of CD69 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	78±127	8±43	42±112	86±131	27±42	55±66
Naïve (IgD+27-)	1±86	1±35	6±53	29±103	4±36	neg
Pre-switch memory (IgD+27+)	246±262	69±58	215±241	184±200	171±216	207±270
Post-switch memory (IgD-27+)	130±144	85±50	156±147	207±243	148±162	138±74
DN (IgD-27-)	108±142	52±63	136±154	119±115	78±65	221±103

Table 6. MFI values of CD86 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	69±136	65±66	111±143	144±102	67±136	95±69
Naïve (IgD+27-)	48±124	12±110	38±152	13±227	13±131	16±95
Pre-switch memory (IgD+27+)	84±184	116±102	161±144	148±175	132±172	98±183
Post-switch memory (IgD-27+)	181±141	226±69	225±159	277±174	261±150	284±187
DN (IgD-27-)	125±90	163±54	169±92	169±89	158±102	110±64

Table 7. MFI values of HLA-DR on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	25596±8709	32803±10483	26643±14333	23869±11973	38693±11768*#	43917±1848*#
Naïve (IgD+27-)	31834±13161	38098±12499	31259±17522	28832±14606	44972±14006*	54894±23771*#
Pre-switch memory (IgD+27+)	27497±13657	32426±9149	23627±10679	25547±13292	32238±12340	44302±21876****
Post-switch memory (IgD-27+)	18012±6990	20544±6344	19199±10276	17262±8251	28359±7472*#	27368±15824
DN (IgD-27-)	22010±9004	27647±9225	22382±12687	21495±10344	33234±11366*	35312±11773*

* $p < 0.05$ in comparison with Controls

# $p < 0.05$ in comparison with MTX pre-bio

3.3. CXCR5 expression is increased since early RA

In all groups studied, no significant differences were found in the frequencies of CXCR5+ B cells in all B cell subpopulations (data not shown). Nevertheless, CXCR5 MFI values were significantly increased in total CD19+ B cells in ERA (p = 0.0276) and established RA patients after treatment with MTX and TNF inhibitors when compared to controls ($p < 0.05$) (Table 8). Moreover, it was observed that CXCR5 MFI values were significantly increased in naïve B cells in ERA and established RA patients treated with MTX, MTX pre-bio and TNF inhibitors in comparison with controls.

Table 8. MFI values of CXCR5 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	3908±1896	5977±2423*	6380±3343*	5383±2556	7655±3352*	4958±2616
Naïve (IgD+27-)	4022±1993	6089±2354*	6746±3499*	6401±3101*	8173±4148*	5551±2598
Pre-switch memory (IgD+27+)	5794±2190	7001±2276	7576±3785	7058±2927	8882±4221	6449±2905
Post-switch memory (IgD-27+)	4401±2804	5203±2656	5212±3618	4675±2366	5962±2964	4101±2223
DN (IgD-27-)	3480±2604	3539±1913	3693±2610	3088±1869	4088±2531	3448±2268

* $p < 0.05$ in comparison with Controls

3.4. CD95 expression increases on memory B cell subpopulations after treatment with TNF inhibitors and TCZ

The analysis of the expression of CD95, a death receptor whose activation leads to apoptosis, revealed that no significant changes occur in CD95 MFI values on total CD19+ B cells in ERA and established RA patients irrespective of the treatment when compared to healthy controls. However, when analyzing B cell subpopulations, it was found that established RA patients treated with TNF inhibitors and TCZ have increased MFI values of CD95 in post-switch memory B cells in comparison to controls, but no significant differences where observed in the remaining B cell subsets ($p < 0.05$) (Table 9).

Table 9. MFI values of CD95 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19	453±322	231±176	434±319	371±377	585±244	821±495
Naïve (IgD+27-)	313±438	123±263	179±318	99±219	372±224	549±489
Pre-switch memory (IgD+27+)	672±339	644±304	791±426	528±396	1020±458	1052±425
Post-switch memory (IgD-27+)	884±472	1005±470	919±796	981±740	2048±1693*	3243±2950*
DN (IgD-27-)	548±314	587±332	589±342	580±551	796±346	926±641

* $p < 0.05$ in comparison with Controls

3.5. IgM expression and the frequency of IgM+ B cells in circulation in ERA and established RA patients is similar to controls

In all groups of patients studied, no statistically significant differences were observed in the frequency of circulating IgM+ B cells (data not shown). Furthermore, no significant differences were found in IgM MFI values on total CD19+ B cells and all B cell subpopulations analyzed in all groups of patients when compared to healthy controls (Table 10).

Table 10. MFI values of IgM on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19	70±253	111±115	157±212	175±175	152±187	65±188
Naïve (IgD+27-)	67±369	86±178	126±315	191±319	182±235	34±286
Pre-switch memory (IgD+27+)	523±515	532±418	679±676	569±390	744±613	483±588
Post-switch memory (IgD-27+)	74±71	56±51	55±47	91±49	66±37	39±36
DN (IgD-27-)	65±34	46±25	47±46	70±39	40±29	43±25

3.6. ERA and established RA patients have alterations in the expression levels of CD5 and in the frequency of circulating CD5+ B cells

A significant decrease in the frequency of circulating CD5+ B cells was found in total CD19+ B cells, namely on naïve B cells, in all patients´ groups (ERA, established RA treated with MTX, MTX pre-bio, TNF inhibitors and TCZ) in comparison with controls (data not shown). However, the analysis of CD5 expression on B cell subpopulations has shown that both TNF inhibitors and TCZ treatment induce a significant increase in CD5 MFI values on pre-switch and post-switch memory B cells when compared to healthy controls ($p < 0.05$) (Table 10). Furthermore, significantly increased CD5 MFI values were also found on naïve B cells from TCZ treated RA patients in comparison with MTX and MTX pre-bio groups ($p < 0.05$).

Table 11. MFI values of CD5 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19+	615±324	600±348	458±355	461±536	691±201	798±344
Naïve (IgD+27-)	671±382	569±367	483±392	561±607	847±415	1112±224*#&
Pre-switch memory **(IgD+27+)**	695±360	1136±696	822±555	822±852	1530±1012*	1930±1290*#
Post-switch memory **(IgD-27+)**	284±291	532±393	406±297	391±475	643±344*	609±417*
DN (IgD-27-)	447±475	559±279	384±270	341±391	599±333	577±395

* $p < 0.05$ in comparison with Controls

# $p < 0.05$ in comparison with MTX pre-bio

& $p < 0.05$ in comparison with MTX

3.7. TLR9 expression increases after TCZ treatment, but not after MTX or TNF inhibitors

A significant increase in TLR9 MFI values was found in total CD19+ B cells and all B cell subpopulations studied in established RA patients after treatment with TCZ in comparison not only with controls ($p < 0.05$), but also with MTX pre-bio group ($p < 0.05$) (Table 12). In addition, TLR9 MFI was significantly increased on post-switch memory and DN B cells in MTX treated patients when compared to controls. No significant differences were found in ERA or established RA patients treated with MTX pre-bio or TNF inhibitors in comparison with controls in all B cell subsets analyzed.

Table 12. MFI values of TLR9 on B cell subpopulations. Represented values are mean ± SD.

	Controls	ERA	MTX	MTX pre bio	TNF	TCZ
CD19	3333±2104	3698±2759	6072±4023	3602±3751	5885±4692	11968±7361*#
Naïve (IgD+27-)	2900±1819	3143±2165	5207±3440	3417±3756	5473±4509	10051±7456*#
Pre-switch memory IgD+27+)	4833±3068	5135±3458	8340±5231	5516±6265	7239±5515	13837±8414*#
Post-switch memory (IgD-27+)	3964±2373	4730±3544	6913±4226*	4826±5136	6904±5402	11160±5744*#
DN (IgD-27-)	3231±1745	3913±2664	6347±4362*	4110±4603	5915±4918	10126±5732*#

* $p < 0.05$ in comparison with Controls

# $p < 0.05$ in comparison with MTX pre-bio

4. B cell markers and effect of TNF inhibitors and TCZ treatment in comparison with baseline

In order to understand the effect of treatment options before and after RA patients initiate treatment, a second blood collection was performed in established RA patients from MTX pre-bio group after a mean period of 8 months of treatment with TNF inhibitors (n=10) or TCZ (n=7). The MFI values of all cellular markers studied (BAFF-R, TACI, BCMA, HLA-DR, CD69, CD86, CXCR5, CD95, IgM, CD5 and TLR9) were analyzed after treatment and compared to baseline values.

4.1. TNF inhibitors and TCZ treatment affect B cell expression levels of HLA-DR, CD86 and CD95

It was observed that both TNF inhibitors and TCZ treatments affected the expression levels of HLA-DR on B cells. In fact, it was found that after treatment with TNF inhibitors, the HLA-DR MFI values were significantly increased in total CD19+ B cells (p=0.0273) and in all B cell subpopulations analyzed ($p < 0.05$) (Table 13). The same result was observed after treatment with TCZ in total CD19+ B cells, particularly in naïve B cells ($p = 0.0313$) (Table 14). Furthermore, TNF inhibitors also induced a significant increase in CD95 MFI values in total CD19+ B cells ($p = 0.0039$) and in all B cell subpopulations studied ($p < 0.05$) (Table 15). The expression of CD86 was also found to be significantly decreased in total CD19+ B cells (p =0.0371), namely on naïve B cells, after treatment with TNF inhibitors (Table 16). Moreover, no significant differences were observed in the MFI values of BAFF-R, TACI,

BCMA, CD69, CXCR5, IgM, CD5 and TLR9 cell markers in all B cell subpopulations after treatment with TNF inhibitors and TCZ (data not shown).

Table 13. MFI values of HLA-DR on B cell subpopulations before and after TNF inhibitors. Represented values are mean ± SD.

	Before TNF inhibitor	**After TNF inhibitor**
CD19	21399±9378	37743±11951*
Naïve (IgD+27-)	24916±12819	43676±14051*
Pre-switch memory (IgD+27+)	22260±10619	31610±12821*
Post-switch memory (IgD-27+)	16126±6740	27972±7759*
DN (IgD-27-)	18895±7880	31763±10821*

* $p < 0.05$ in comparison with established RA patients before TNF inhibitors

Table 14. MFI values of HLA-DR on B cell subpopulations before and after TCZ. Represented values are mean ± SD.

	Before TCZ	**After TCZ**
CD19	21118±13439	49510±15320*
Naïve (IgD+27-)	30610±18731	62972±18502*
Pre-switch memory (IgD+27+)	27221±17445	50681±20332
Post-switch memory (IgD-27+)	16649±11418	29879±17053
DN (IgD-27-)	18435±11920	38846±23605

* $p < 0.05$ in comparison with established RA patients before TCZ

Table 15. MFI values of CD95 on B cell subpopulations before and after TNF inhibitors. Represented values are mean ± SD.

	Before TNF inhibitor	**After TNF inhibitor**
CD19	203±263	639±182*
Naïve (IgD+27-)	90±190	417±184*
Pre-switch memory (IgD+27+)	352±323	1111±378*
Post-switch memory (IgD-27+)	655±511	1718±731*
DN (IgD-27-)	357±455	876±248*

* $p < 0.05$ in comparison with established RA patients before TNF inhibitors

Table 16. MFI values of CD86 on B cell subpopulations before and after TNF inhibitors. Represented values are mean ± SD.

	Before TNF inhibitor	**After TNF-inhibitor**
CD19	157±68	69±137*
Naïve (IgD+27-)	70±117	Neg*
Pre-switch memory (IgD+27+)	190±105	130±172
Post-switch memory (IgD-27+)	256±122	269±151
DN (IgD-27-)	161±78	164±102

* $p < 0.05$ in comparison with established RA patients before TNF inhibitors

5. Quantification of B cell cytokines and chemokines

To analyze the cytokine and chemokine environment in circulation directly related with B cell activation, the serum levels of sCD23, a marker of B cell maturation [123-125] and CXCL13, an important B cell chemokine [126-128] were determined by ELISA in serum samples from all patients and healthy controls.

5.1. ERA patients, but not established RA, have higher sCD23 levels in circulation

It was observed that sCD23 levels were significantly higher in ERA patients when compared to healthy controls (p=0.0240) and established RA patients treated with MTX pre-bio (p=0.0147) (Figure 5). Furthermore, no significant differences were found in sCD23 levels in all established RA patients irrespective of treatment in comparison with controls.

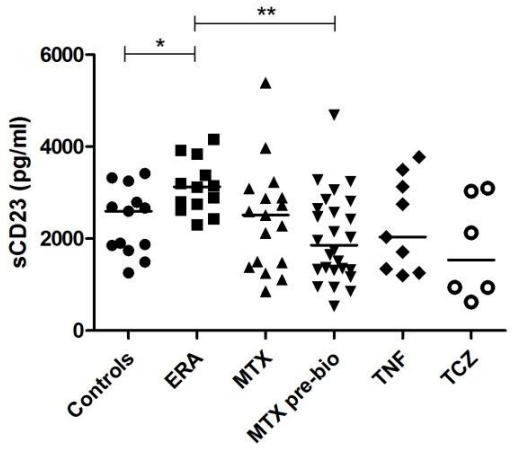

Figure 5. sCD23 serum levels are increased in ERA patients, but not in established RA irrespective of treatment; * $p < 0.05$ in comparison with Controls, ** $p < 0.05$ in comparison with ERA.

5.2. Serum CXCL13 levels are increased in ERA and established RA patients

The B cell chemokine CXCL13 was significantly increased in ERA (p=0.0018) and established RA patients treated with MTX (p=0.0075), MTX pre-bio (p=0.0006) and TNF inhibitors (p=0.0326) when compared to healthy controls (Figure 6). However, no significant differences were observed in CXCL13 serum levels in established RA patients after TCZ treatment when compared to controls.

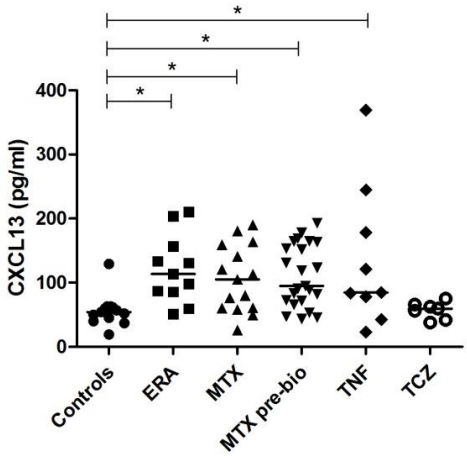

Figure 6. CXCL13 serum levels are increased in ERA and established RA patients treated with MTX, MTX pre-bio and TNF inhibitors; * $p < 0.05$ in comparison with Controls.

V. Discussion and Conclusions

Several studies have reported the importance of B cells in RA pathogenesis through diverse mechanisms [13, 129, 130]. B cells can produce autoantibodies (RF and anti-CCP), which can form immune complexes that deposit in the joints, causing inflammation; function as APCs and activate T cells; release cytokines once activated and participate in ectopic lymphoid neogenesis [13, 15, 23, 71, 76, 85]. In the present study, an extensive characterization of B cell phenotype was performed in untreated early RA patients with less than 1 year of disease duration and compared to established treated RA patients and healthy individuals.

Abnormalities in the distribution of peripheral blood B cells have been previously reported in RA patients [120, 131], but there is still no consensus about this subject. In fact, while some studies have reported a decrease in memory B cells in circulation, other works have found an increase or no changes in the frequencies of circulating B cells, particularly memory B cell subsets, not only in RA but also in other autoimmune conditions [120, 131-135].

The human memory B cell population is heterogeneous, comprising mutated pre-switch IgD+CD27+, post-switch IgD-CD27+ and double negative IgD-CD27- memory B cells [136, 137]. It is widely accepted that post-switch IgD-CD27+ memory B cells are post-GC highly mutated memory B cells [137, 138]. Previous studies by our group have reported that very early RA patients (with less than 6 weeks of disease duration) have lower frequencies of circulating pre-switch IgD+CD27+ memory B cells [120] an unclass-switched memory B cell subset that plays a crucial role in early immune responses by producing high-affinity IgM. Of note, it has been demonstrated that memory B cells accumulate in the synovial membrane of RA patients, suggesting that the accumulation of pre-switch memory cells within inflamed tissue may contribute to a decrease in peripheral blood [120, 131]. Other studies have found that RA patients with long-standing disease have increased circulating levels of post-switch memory B cells and that this memory B cell subset also highly infiltrates synovial membranes in RA [131], which was suggested to be associated with a persistent immunological stimulation that would be enough to over-compensate the enhanced sequestration of these cells in the synovium.

In the present study, although no differences were found in the frequencies of total CD19+ B cells in circulation, it was observed that established RA patients treated with MTX and MTX pre-bio had higher frequencies of circulating double negative (IgD-CD27-) B cells when compared to healthy controls. These changes can be related either with the effect of MTX treatment and/ or with disease activity, or disease duration [134, 139]

To better characterize the B cell phenotype in early and established RA patients, several cellular markers were studied which were directly related with B cell activation through BAFF (BAFF-R, TACI and BCMA); cellular activation (HLA-DR, CD69 and CD86); B cell chemotaxis (CXCR5); Fas-mediated apoptosis (CD95); BCR-mediated activation (IgM); B cell differentiation and maturation (CD5); and activation mediated by TLRs (TLR9).

BAFF is a fundamental cell survivor factor essential for B cell proliferation and activation, and it has been suggested that this cytokine may be a potential target in B cell-related auto-immune diseases. BAFF binds to 3 receptors: BAFF-R, TACI and BCMA, but the precise mechanisms that control the expression of all 3 BAFF receptors are currently unclear [140]. Previous studies by our group have demonstrated that BAFF levels are increased in RA patients in serum and locally in the joints in synovial fluid [141]. Furthermore, it has also been demonstrated that BAFF-R and TACI gene expression increase with disease progression [142, 143]. Nevertheless, these results were obtained at the gene level, not at the protein level. In the present work, no significant differences were detected in the expression levels of both BAFF-R and BCMA in all groups, but TACI expression was increased in total CD19+ B cells, particularly in post-switch memory B cells, in established RA after treatment with TNF inhibitors. The results observed for BAFF-R are in accordance with previous studies, where no differences were detected in comparison with healthy controls [108]. Studies with knockout mice have shown that B cell proliferation depends on either BAFF-R or TACI signaling. BAFF-R signaling can result not only in survival enhancement, but also in B cell maturation, while TACI triggering can stimulate class-switching and plasma cell differentiation [144, 145]. However, the functional activity of TACI is ambiguous [145, 146]. TACI provides positive signals driving T-independent B cell responses, but also delivers negative signals suppressing B cell activation [143, 147]. It is possible that the increased TACI expression observed after anti-TNF treatment results from a direct effect of the treatment. Increased TACI could deliver inhibitory signals to activated B cells, namely post-switch memory B cells. Since BAFF binds with higher affinity to BAFF-R, leading to B cell activation [143] the increased levels of TACI could compete for BAFF-binding and thus induce an inhibitory effect on B cell activation as a consequence of anti-TNF treatment. Moreover, the normal expression levels observed for BCMA in all groups analyzed can be related to the fact that this receptor is mainly expressed at later stages of B cell differentiation, namely by plasma cells [148, 149].

Previous studies have reported that B cells once activated increase the expression of activation markers such as HLA-DR, CD69 and CD86 [100, 150], which are expressed in small amounts or not at all on resting B cells. In the present work, although no differences were detected in CD69 and CD86 expression levels in all B cell subpopulations in all groups

analyzed, HLA-DR expression was increased in established RA patients after treatment with TNF inhibitors and TCZ. It has been demonstrated that treatment options administrated to RA patients are able not only to ameliorate clinical symptoms, but also to diminish the cellular infiltration in the joints [55, 60, 151]. It is possible that due to the effect of anti-TNF and TCZ treatments, activated B cells infiltrating the synovial membrane of established RA patients return to the peripheral blood and re-circulate. These activated B cells express higher levels of HLA-DR, in accordance with their function as APCs locally in the joints [12, 30, 152]. Nonetheless, the absence of differences in CD69, an early activation marker [153], and CD86, a costimulatory molecule important for B-T cell interactions [154] [155], in all studied groups might indicate that the activation state of peripheral blood B cells is lower than in B cells recruited to the joints, where the main inflammatory process occurs in RA. Furthermore, the decreased CD86 expression observed in established RA patients treated with TNF inhibitors when compared to baseline levels further supports an effect of anti-TNF therapies in inhibiting B cell activation state, which is in accordance with previous studies [156]

Apoptosis through engagement of the death receptor Fas (CD95) plays pivotal roles in maintaining peripheral immune self-tolerance [157]. Importantly, defects in apoptosis, namely in the CD95-dependent death receptor pathway, have been demonstrated to occur in RA pathogenesis [158, 159]. The increased CD95 MFI values observed in post-switch memory B cells in established RA patients after treatment with TNF inhibitors and TCZ when compared to controls are probably a direct consequence of treatment as a mean to counteract B cell activation and autoimmunity. Thus, a higher level of expression of CD95 after treatment with anti-TNF and/ or TCZ, turns B cells more prone to apoptosis, which is in accordance with recent reports [160]. Since this observation was specifically detected in post-switch memory B cells, this supports the relevance of this B cell subpopulation in RA pathogenesis as previously demonstrated [131]. Furthermore, the increase in CD95 expression levels in all B cell subpopulations in established RA patients after treatment with TNF inhibitors when compared to baseline levels are in accordance with recent reports that suggest that TNF inhibitors have the ability to modulate Fas-mediated apoptosis in RA [160].

In this work, decreased frequencies of CD5+ B cells were observed in all groups of RA patients studied in comparison with healthy controls. Previous studies have found alterations in these cells not only in RA, but also in other autoimmune diseases [161-163]. CD5+ B cells are known to be associated with bone resorption through IL-6 production, a cytokine that supports osteoclast differentiation [161, 164, 165]. The decreased frequency of these cells in circulation in all patients' groups could result from a recruitment of CD5+ B cells towards the synovial membrane, where the main inflammatory process occurs in RA, which would

contribute for bone erosion. Furthermore, increased CD5 MFI values were also detected on B cells in established RA patients after treatment with TNF inhibitors and TCZ. CD5 has also been suggested as a negative regulator of B cell activation [166]. Thus, it is possible that the increased expression of CD5 on B cells after anti-TNF and TCZ treatments constitutes a way to counteract the development of autoimmunity. In fact, CD5+ B cells have also been suggested to be precursors of regulatory B cells [167, 168] which further supports the results observed.

B cell stimulation through TLRs can also be a mechanism directly related with autoimmunity [169]. TLR9 recognizes unmethylated microbial CpG DNA, which in turn is similar to certain CpG DNA motifs within the mammalian genome [170, 171]. Supporting the involvement of TLR9 signaling in the development of autoimmunity, hypomethylated genomic CpG DNA has been found in RA and SLE patients [171]. Moreover, it is known that the dual engagement of BCR and TLR9 may activate autoreactive B cells that recognize endogenous CpG DNA released from apoptotic cells and CpG DNA-IgG immune complexes in RA joints, and induce their development into RF-producing B cells [171, 172]. Also, stimulation through TLRs, namely TLR9, can be more efficient than T-dependent pathways at downregulating BAFF-R expression on memory B-cell populations [173]. Of note, it has been demonstrated that TLR7 and TLR9 triggering strongly upregulate TACI expression [121, 173], which suggests that autoimmunity might also be correlated with reciprocal induction and activation of TACI and TLRs [143, 172, 173]. In this study, increased TLR9 expression was observed in all B cell subpopulations in established RA patients after treatment with TCZ when compared to controls. Also, increased TLR9 MFI values were detected on post-switch memory and DN B cells in MTX treated patients in comparison with controls. These observations might indicate that although MTX and TCZ treated RA patients have the lowest disease activity scores, B cells may have escape mechanisms through TLR9-mediated activation to perpetuate autoimmunity.

CXCR5 is a chemokine receptor highly expressed in recirculating B cells, in subsets of CD4+ and CD8+ T cells and monocytes [117, 174-176]. CXCR5 and its ligand, CXCL13, involved in B cell chemotaxis, are upregulated in RA synovium, suggesting a local role in B cell recruitment toward the synovial membrane [117, 174, 175, 177]. In this study, increased CXCR5 expression levels were observed on B cells from untreated ERA and established RA patients after treatment with MTX and TNF inhibitors, which supports B cell recruitment towards the joints since early RA onset. Moreover, the increased serum CXCL13 levels found in all patients groups when compared to controls further corroborate an upregulation of B cell chemotaxis in RA [12, 175, 176].

CD23, the low-affinity Fc receptor for IgE, is a C-type lectin that has a membrane (mCD23) and a soluble form (sCD23) and each form binds to multiple ligands and exerts diverse physiologic functions. During the activation of normal B cells, mCD23 is cleaved by a cell-associated protease and released as sCD23 [123, 124, 178]. Several authors have described increased levels of sCD23 in RA patients and their association with joint erosion [123, 179]. It has been suggested that the increased serum levels of sCD23 in RA patients are due to a higher expression of mCD23 on CD5+ B cells [162]. In this study, the increased serum sCD23 levels detected in untreated ERA patients in comparison with controls can be related with an early B cell and macrophage activation [180].

To sum up, established RA patients have disturbances in the frequencies of memory B cell subpopulations in circulation, namely in double negative IgD-CD27- B cells. Also, the expression levels of cellular markers can be affected by TNF inhibitors and TCZ treatment, particularly activation markers (HLA-DR, CD86). Furthermore, untreated ERA patients have significantly decreased frequencies of CD5+ B cells, elevated CXCR5 expression and higher serum CXCL13 levels in comparison with controls, which supports an early B cell activation in RA pathogenesis as previously suggested [120, 141].

VI. Future Perspectives

In order to expand and further complete this study it would also be important to do some in vitro assays with B cell stimuli (anti-IgM, BAFF, CpG motifs…) using isolated B cell subsets to better characterize B cell subpopulations and their activation pattern. Simultaneously, apoptosis studies could also be performed. Moreover, increasing the cohorts of patients and extending the time between baseline and follow-up blood collection dates would also allow a more robust statistical analysis. It would also be important to study the effects of treatment options individually in particular all TNF inhibitors available (infliximab, adalimumab, certolizumab, golimumab and etarnecept), since it is possible that different drugs can have different effects. Of note, it would also be interesting to study other therapeutic approaches such as the B cell depleting drug rituximab and/ or abatacept, a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4 that inhibits T cell activation. Furthermore, in order to have a more complete discrimination between B cell subpopulations, the use of IgD/ CD38 system classification could be very useful, since it allows the identification of six B cell subpopulations, contrarily to IgD/CD27. Indeed, the conjugation of the results obtained with the two classification systems would allow a more complete analysis of B cell subsets.

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APPENDIX 1

1. 2010 ACR/EULAR classification criteria for rheumatoid arthritis [8]

	Score
Target population (Who should be tested?): Patients who	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA)‡	
A. Joint involvement§	
1 large joint ¶	0
2 – 10 large joints	1
1 – 3 small joints (with or without involvement of large joints) #	2
4 – 10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint) **	5
B. Serology (at least 1 test result is needed for classification)††	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)‡‡	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms§§	
<6 weeks	0
≥6 weeks	1

***** The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfillment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA.

† Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

‡ Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

§ Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are *excluded from assessment*.

Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement.

¶ “Large joints” refers to shoulders, elbows, hips, knees, and ankles.

“Small joints” refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists. ** In this category, at least 1 of the involved joints must be a small joint; the other joints can include 40 any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.).

††Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF.

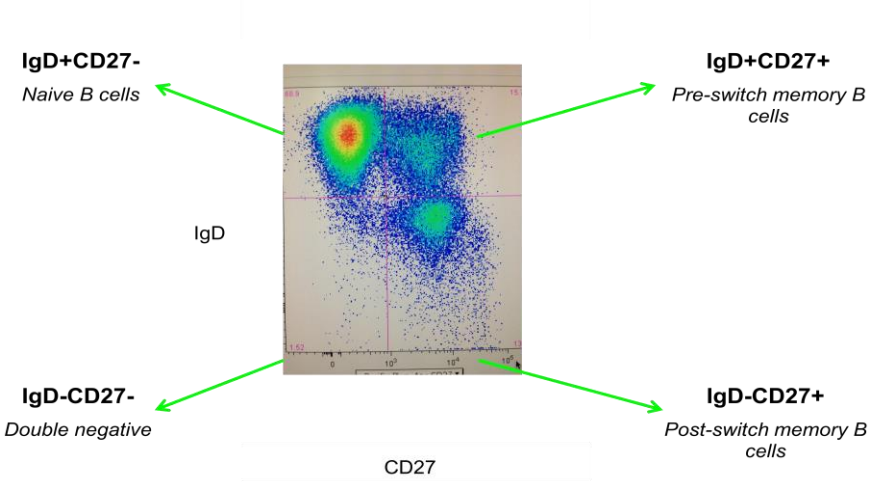
‡‡ Normal/abnormal is determined by local laboratory standards.

§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, welling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

APPENDIX 2

Classification of CD19+ B cell subpopulations

Circulating B cell (CD19+) subpopulations were defined according to IgD and CD27 expression and classified as naïve(IgD+CD27-), pre-switch-memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double-negative (IgD-CD27-) B cells.



Circulating B cell (CD19+) subpopulations were defined according to IgD and CD38 expression and classified as IgD+ memory (IgD+CD38-), naïve (IgD+38+), transitional (IgD+CD38++), resting memory (IgD-CD38-), post-GC memory (IgD-CD38+) B cells and plasmablasts (IgD-CD38++). In this study this classification was only used to analyze plasmablasts (IgD-CD38++).